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Expressions of p53 and PCNA Do Not Correlate With the International Index or Early Response to Chemotherapy in Non-Hodgkin's Lymphoma

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The expression of p53 and PCNA on deparaffinized sections of tumor was assessed in relation to the International Index and response to chemotherapy. Thirty-five non-Hodgkin's lymphoma (NHL) patients were divided into three groups: aggressive NHL, mantle cell lymphoma (MCL), and low-grade NHL. None of the expressions correlated with the International Index or early response to chemotherapy in any group. In low-grade NHL, none of the patients expressed p53. Only one of three patients with overexpression of p53 showed conformational change and alteration of sequence in exon 7 by PCR-SSCP and DNA sequencing. The results showed that p53 and PCNA staining were not useful for predicting early response to chemotherapy, and that their expressions had no correlation with the International Index. Am. J. Hematol. 58:42–48, 1998. © 1998 Wiley-Liss, Inc.

Key words: p53; PCNA; the International Index; PCR-SSCP; non-Hodgkin's lymphoma

INTRODUCTION

The presence of a mutation of the p53 gene and the overexpression of mutated p53 proteins are factors in the tumorigenesis of several neoplasms. In lymphoid neoplasms, p53 mutations are found mainly in aggressive B-cell non-Hodgkin's lymphomas (NHL) [1], and are suspected to be associated with histological transformation of follicular lymphoma [2,3]. Furthermore, p53 overexpression is a poor prognosis marker in mantle cell lymphoma [4] or high-grade B-cell lymphomas [5]. Immunohistochemistry for p53 cannot directly detect mutations, but mutated protein can be detected by immunostaining because most missense mutations prolong the half-life of the protein [6]. Hodgkin's disease (HD) showed a high proportion of p53 positive cells among Reed-Sternberg (RS) and Hodgkin's cells [7]. Similarly, CD30 (Ki-1)-positive, anaplastic large cell lymphomas (ALCL) frequently overexpress p53; however, their p53 overexpression does not correlate with p53 gene mutations [8]. Besides, there is some doubt as to whether p53 expression correlates with the presence of mutation of the p53 gene [9]. Proliferating cell nuclear antigen (PCNA) is a 36-kD non-histone nuclear polypeptide expressed primarily in S-phase cycling cells. In normal human cells, PCNA exists in a complex with a cyclin, cyclin-dependent protein kinase (CDK) and p21 induced by p53 [10]. PCNA is a factor in DNA replication, but it is inhibited by p21 [11]. The proliferative activity of PCNA has been evaluated in NHL [12], and found to correlate with Ki-67 score, S-phase fraction, histological grade, and prognosis. And in a study on carcinoma, high expression of PCNA correlated with histologic grade and p53 overexpression in breast carcinoma [13], and PCNA expression differed significantly between tumors with

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TABLE I. Clinical Features of the Patients With Aggressive NHL*

Patient no.	Sex/age	International Index	Pathology[d]	Expression of p53	Expression of PCNA	Response	Survival[a]
1	M/62	L (1)	IBL (B)	(−)	(−)	CR	12+
2	M/79	L (1)	IBL (B)	(−)	(++)	CR	6+
3	F/44	L (1)	DL (B)	(++)[b]	(+)	CR	22− (Relapse)
4	M/62	L (1)	DM (B)	(−)	(−)	CR	6+
5[c]	M/38	L (1)	ALCL	(++)	(++)	PR	7− (Heart failure)
6	M/66	L (1)	SNC (Burkitt)	(+++)	(−)	CR	10+
7	M/60	LI (2)	IBL (B)	(−)	(+++)	CR	10+
8[c]	M/74	LI (2)	IBL (B)	(−)	(+)	PR	10+
9	M/54	LI (2)	IBL (T)	(++)	(++)	CR	9+ (Relapse)
10	F/41	HI (3)	IBL (B)	(+)	(++)	CR	16+
11	M/66	HI (3)	IBL (B)	(−)	(+)	Death	
12	M/55	HI (3)	IBL (B)	(−)	(−)	PR	6− (Heart failure)
13	M/52	HI (3)	DM (B)	(++)	(++)	PR	9+
14[c]	M/84	HI (3)	IBL (B)	(+)	(++)	PR	16+
15	M/56	H (4)	DL (B)	(−)	(++)	CR	10+
16	F/67	H (4)	IBL (T)	(−)	(++)	CR	30+
17	M/69	H (4)	DM (B)	(−)	(−)	CR	13+
18[c]	M/68	H (4)	DSC (B)	(−)	(+)	CR	7+
19	M/71	H (5)	DM (B)	(+)	(+++)	CR	12+
20	M/75	H (5)	DSC (B)	(−)	(+++)	Death	
21	M/71	H (5)	ALCL	(+++)[b]	(+++)	PD	4−
22	F/79	H (4)	SNC	(+++)	(−)	PD	6−

*L, Numbers of risk factors 0–1; LI, 2; HI, 3; H, 4–5. CR, complete remission; PR, partial remission; PD, progressive disease.
[a]Survival in months from diagnosis; +, alive, −, expired.
[b]Conformational change was examined by PCR-SSCP.
[c]Relapsed case.
[d]IBL, immunoblastic; DL, diffuse large cell; DM, diffused mixed small and large cell; ALCL, anaplastic large cell lymphoma; SNC, small non-cleaved cell; DSC, diffuse small cleaved cell; (B), B-cell type; (T) T-cell type.

poor and good histopathological response to radiation therapy in bladder cancer [14]. In fact, various factors have been studied to predict the prognosis of non-Hodgkin's lymphoma. Recently, an International Index was designed to aid in the selection of appropriate therapies for patients with aggressive non-Hodgkin's lymphoma [15]. This system divides patients into four groups based on independent prognostic factors (age, lactate dehydrogenase level, performance status, stage, and extranodal disease sites) and showed a clear difference in long-time survival among the groups. Furthermore, another study indicated the applicability of its index to low-grade NHL [16]. In the present study, we compared the expression of p53 and PCNA with the International Index and with early response to chemotherapy in NHL, to evaluate whether immunohistochemistry supports the classifications with the new Index.

MATERIALS AND METHODS

Patients and Samples

Among lymphoma patients treated between 1993 and 1995 at Hiroshima University Hospital, Ohtake National Hospital and Hiroshima Red Cross & Atomic-Bomb Survivors Hospital, 35 patients (24 male, 11 female; mean age 66 years) with fresh and relapsed non-Hodgkin's lymphomas (NHL) were selected at suitable for p53, PCNA staining, and treated with our chemotherapy protocol. Histological and clinical features are indicated in Table 1. Except for anaplastic large cell lymphoma (ALCL) and mantle cell lymphoma (MCL) recognized by REAL classification [17], all pathological diagnoses were based on the Working Formulation (WF). Eight patients with large cell immunoblastic (plasmacytoid) (IBL-B), 2 patients with large cell immunoblastic (clear cell) (IBL-T), 2 patients with diffuse large cell (DL), 2 patients with diffuse small cleaved cell (DSC), 2 patients with diffuse mixed small and large cell (DM), 2 patients with small non-cleaved cell (SNC), 3 patients with small lymphocytic (SL), 3 patients with follicular small cleaved cell (FSC), and 3 patients with follicular mixed small cleaved and large cell (FM) lymphoma were included in the study. On admission, the International Index was calculated by the International Non-Hodgkin's Lymphoma Prognostic Factors Project based on age, tumor stage, serum lactate dehydrogenase (LDH) concentration, performance status, and number of extranodal disease sites [15]. Then patients were assigned to one of four risk groups on the basis of the number of presenting risk factors: 0 or 1, low risk; 2, low intermediate risk; 3, high intermediate risk; 4 or 5, high risk (Table I). All patients were treated with CHOP or a more intensive

TABLE II. Clinical Features of the Patients With MCL, Low-Grade NHL*

Patient no.	Sex/age	International Index	Pathology[d]	Expression of p53	Expression of PCNA	Response	Survival[a]
23[c]	M/36	LI (2)	MCL	(++)[b]	(++)	PR	24+
24	M/61	LI (2)	MCL	(+)	(+)	PR	15− (PD)
25	F/78	HI (3)	MCL	(−)	(−)	PR	19+
26	M/77	H (5)	MCL	(−)	(−)	CR	6− (Pneumonia)
27[c]	F/36	L (0)	FSC (B)	(−)	(−)	CR	10+
28	F/36	L (1)	FM (B)	(−)	(+++)	CR	6+
29	F/50	L (1)	FSC (B)	(−)	(+)	PR	6+
30	F/40	L (1)	FSC (B)	(−)	(+)	PR	6+
31	M/72	LI (2)	SL (B)	(−)	(−)	PR	15+
32[c]	M/57	HI (3)	SL (B)	(−)	(−)	PD	15+
33	M/57	H (4)	SL (B)	(−)	(++)	PR	7+
34	F/80	H (4)	FM (B)	(−)	(−)	PR	9+
35	F/83	H (4)	FM (B)	(−)	(++)	PR	6+

*L, Numbers of risk factors 0–1; LI, 2; HI, 3; H, 4–5. CR, complete remission; PR, partial remission; PD, progressive disease.
[a]Survival in months from diagnosis; +, alive, −, expired.
[b]Conformational change was examined by PCR-SSCP.
[c]Relapsed case.
[d]See footnote d in Table I.

protocol composed of an alternating anthracyclin-containing regimen. Both protocols were modified for elderly subjects (more than 69 years old). The response to the chemotherapy was assessed after three courses of CHOP or induction therapy of the protocol according to the WHO handbook for standardized cancer registries.

Immunohistochemistry

Immunohistochemical analysis was performed by the alkaline phosphatase/anti-alkaline phosphatase (APAAP) technique and avidin-biotin methods using p53 (Dako, Carpinteria, CA; DO-7) and PCNA (Dako, PC10) on deparaffinized sections. An antigen retrieval technique with boiling in 10 mM citrate buffer was used before the application of the anti-p53 antibody. Tumors were considered positive for p53 and PCNA expression if the neoplastic cells exhibited nuclear staining greater than 3%. Cells with greater than 3% and less than 10% nuclear staining were considered single-positive (+), greater than 10% and less than 30%, double-positive (++), and greater than 30%, triple-positive (+++).

Polymerase Chain Reaction Single-Strand Conformation Polymorphism (PCR-SSCP)

High molecular weight DNA was isolated by standard procedures from lymph nodes of three patients. PCR was performed by standard methods using oligonucleotide primers to amplify exon 5 through 8 [18], where the majority of p53 mutations occur.

Direct DNA Sequencing

DNA sequencing of exons was performed for one patient who showed conformational change of exon 7 by SSCP analysis.

Statistical Analysis

Significant differences were calculated using the Student's *t*-test or χ^2 test. A threshold *P* value less than 0.05 was considered significant.

RESULTS

Patient Characteristics and International Index Distribution

The International Index of the International Non-Hodgkin'e Lymphoma Prognostic Factors Project was designed for aggressive non-Hodgkin's lymphoma [15]. First, we examined the relation between the expression of p53 and PCNA, and both the Index, and early response to chemotherapy in intermediate-grade NHL, high-grade NHL, and ALCL. In this study, the above three types were considered aggressive NHLs. Second, we applied the Index to nine cases of low-grade NHL and four cases of MCL. MCL is a distinctive type of follicular lymphoma [19], with clinical features similar to that of low-grade NHL [20].

The distribution of pathological features among the International Index varied in aggressive NHLs: six patients with Low, 3 patients with Low-intermediate, 5 patients with High-intermediate, and 8 patients with High risk (Table I). Similarly, there was no prominent distribution among the International Index in low-grade NHLs and MCL (Table II).

p53, PCNA Immunohistochemistry

All patients with low-grade NHL were p53-negative (Table II). One of these patients showed p53-positivity of less than 3% and a follicular architectural growth pattern (Fig. 1). Among the aggressive NHLs, p53-positivity was

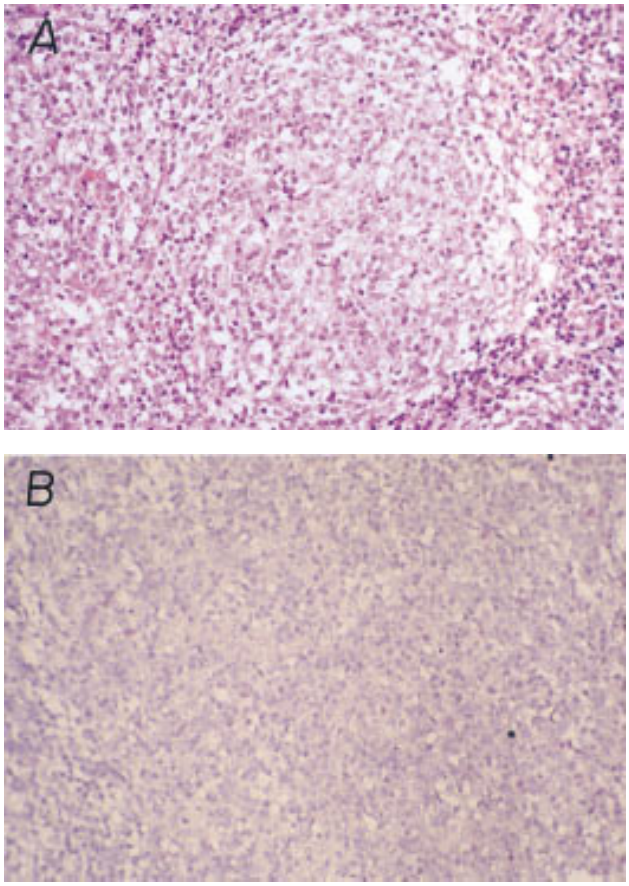

Fig. 1. The patient (no. 28) diagnosed as FM. Lymph node biopsy showed follicular mixed, small cleaved and large (A). The overall negative staining of p53 using DO7 (DAKO) in the lymph node biopsy (B).

detected in 3/6 Low, 1/3 Low-intermediate, 3/5 High-intermediate, and 3/8 High risk patients. High-risk patients showed no tendency for increased p53-positivity ($P = 0.958$) (see Fig. 3).

One patient (n. 23) was diagnosed retrospectively as MCL with a follicular pattern by reviewing a sample taken 10 years prior. The patient relapsed as diffuse lymphoma after 10 years of complete remission. Tumor cells at onset were p53-negative, but at the time of relapse were p53-positive (++), PCNA-positive (++), and showed a diffuse architectural pattern (Fig. 2).

PCNA-positivity was detected in 3/6 Low, 3/3 Low-intermediate, 4/5 High-intermediate, and 6/8 High-risk aggressive NHLs (Table 1). In MCL and low-grade NHL, 2/4 and 5/9, respectively, were PCNA positive (Table II). There was no relation between the International Index and the intensity of PCNA expression ($P = 0.344$) (Fig. 3). Both the small non-cleaved NHL patients were strongly p53-positive (+++) but PCNA-negative, while both the ALCL patients were strongly p53 (++ or +++) and PCNA (++ or +++) positive. Interestingly, two

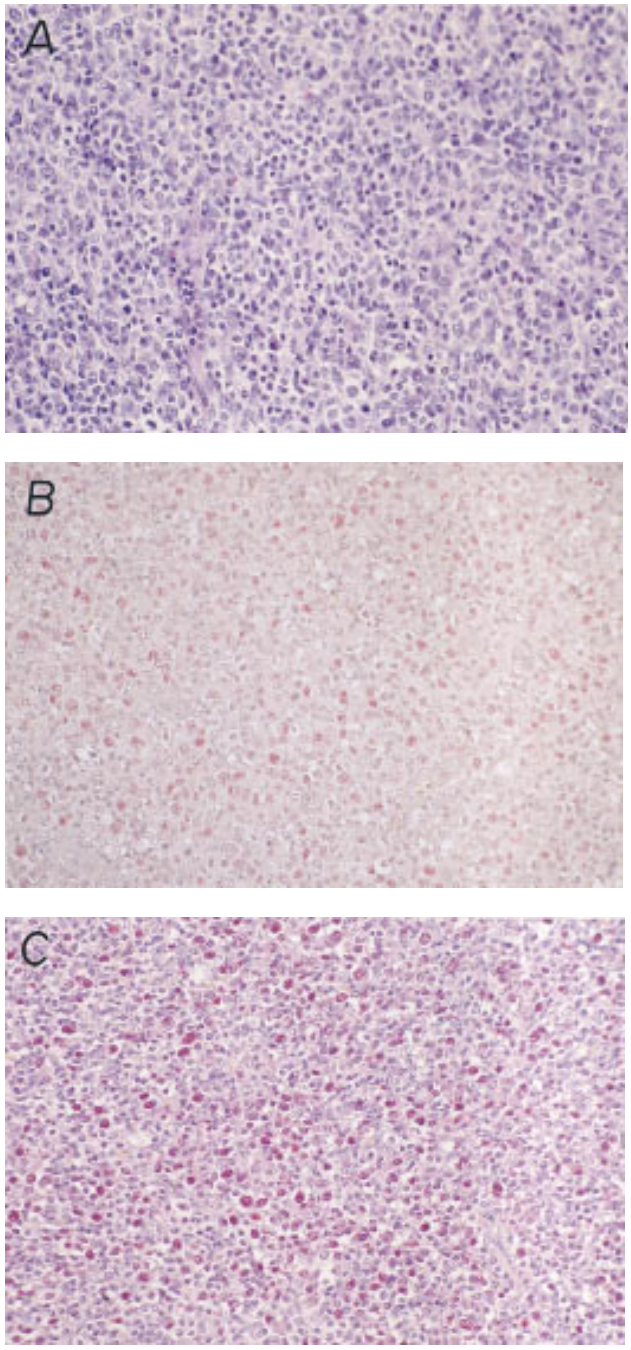

Fig. 2. The patient (no. 23) diagnosed as MCL at the time of relapse. Lymph node biopsy showed MCL, diffuse type (A). p53 immunostaining using DO7 (DAKO) showed numerous cells with p53 overexpression in their nuclei (B). PCNA immunostaining using PC10 (DAKO) showed numerous cells with PCNA overexpression in their nuclei (C).

HD patients were also strongly positive for p53 and PCNA (data not shown).

Early Response to Chemotherapy

We assessed the early response to chemotherapy after three courses of CHOP or induction therapy. Two pa-

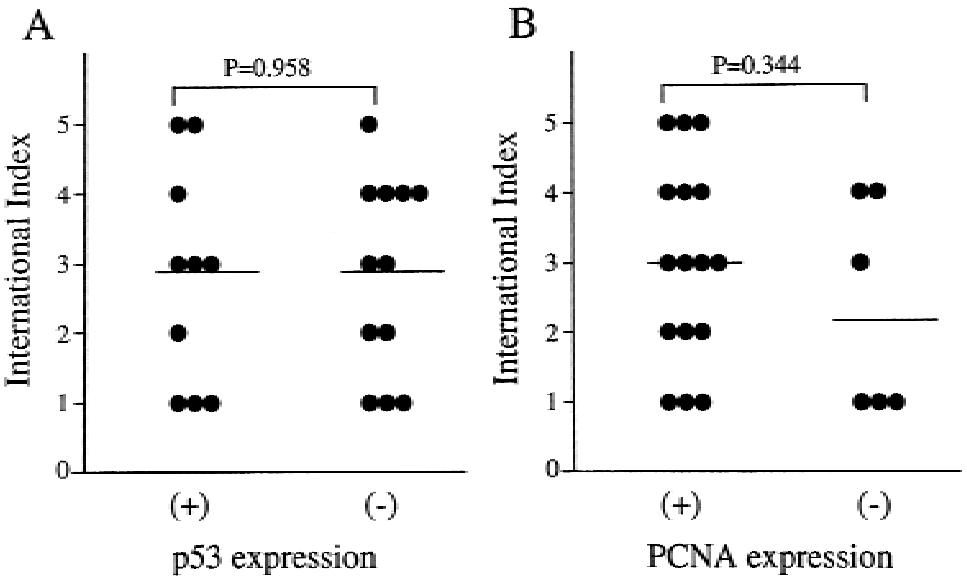

Fig. 3. The relation between the p53 expression (A) or PCNA expression (B) and the International Index. The means of the International Index are indicated as horizontal bars. There is no significant relation, respectively (*P* = 0.958 and *P* = 0.344).

TABLE III. Response to Chemotherapy and Expression of p53 or PCNA in Aggressive Lymphoma

	p53 expression			PCNA expression		
	Negative (n = 12)	Positive (n = 10)	*P* value*	Negative (n = 6)	Positive (n = 16)	*P* value*
Response to chemotherapy			0.721			1.000
CR	8	5		4	9	
Non-CR	4	5		2	7	

*Statistical analysis was performed utilizing χ^2 test.

tients died of heart failure during chemotherapy. The ratio of partial remission (PR) and progressive disease (PD) or complete remission (CR) between the p53-positive and p53-negative patients did not differ significantly among aggressive NHLs ($P = 0.721$) (Table III). Similarly, there was no significant difference in chemotherapy response between the PCNA-positive and PCNA-negative patients ($P = 1.000$) (Table III). Low-grade NHL was not assessed, because all 9 patients were p53-negative and only 2 patients entered CR. Among MCL, only 1 patient, with p53 and PCNA-negative, entered CR (Table II).

PCR-SSCP and Sequencing Results

The immunostaining of p53 may result in a prolonged half-life of the mutated protein arising from conformational changes to the gene [21]. The three aggressive NHLs (nos. 3, 21, 23) with strong p53 positivity (++ or +++) were examined for p53 gene mutations in exons 5 through 8. The sample for patient no. 3 was obtained at relapse. In one patient (no. 23), a conformational alteration was found in exon 7 (Fig. 4). DNA sequencing analysis showed that the change occurred at codon 240 (TGA→TGC; Ser→Arg) (Fig. 5).

DISCUSSION

In this study, we defined ALCL (REAL classification) and intermediate- and high-grade NHL (International Working Formulation) as aggressive lymphoma, and scored the prognostic risk factors to identify an International Index in these patients. Furthermore, we applied the International Index to patients with MCL and low-grade lymphoma; one study had showed that it was useful for such cases [16]. For the evaluation of immunohistochemistry and response to chemotherapy, however, we separated MCL and low-grade lymphoma from aggressive lymphoma (Tables I, II).

The immunohistochemical overexpression of bcl-2 protein or cell cycle related proteins such as p53, p21, mdm 2, and cyclin D_1 have been analyzed whether they made sense in non-Hodgkin's lymphoma. Although the detection of p53 protein by immunostaining of paraffin-embedded tissue does not always correlate with conformational changes to the p53 gene, the technique is easier than PCR-SSCP and fast. While on the one hand, one report on colorectal cancer showed a very close correlation between overexpression of p53 protein and mutation of p53 gene [22], on the other hand, another report dem-

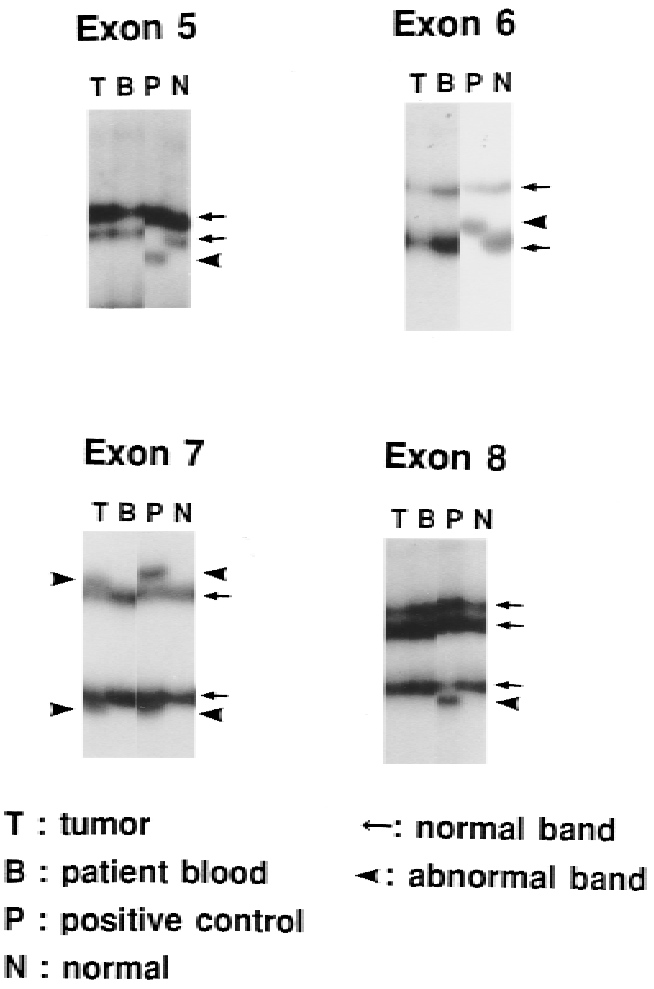

Fig. 4. PCR-SSCP analysis of p53 gene. Conformational change in exon 7 was identified in the patient (no. 23).

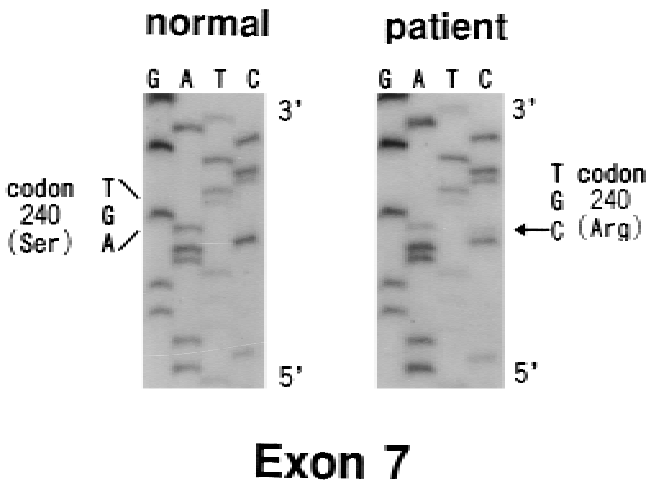

Fig. 5. DNA sequencing. The patient showed a single-based substitution in codon 240(TGA→TGC; Ser→Arg) in exon 7.

onstrated the expression of p53 protein in non-Hodgkin's lymphoma was not always dependent on p53 gene mutations [23]. This difference might be due to the frequency of the alterations of p53 gene between carcinoma and hematological malignancies. Irrespective of p53 gene mutations, if p53 overexpression is an indicator of low response to chemotherapy or poor prognosis, an appropriate therapy could be selected for each patient at diagnosis. In the present study, one MCL patient with a p53 gene mutation and p53 overexpression responded poorly to chemotherapy. Since the follow-up period in this study was short, we evaluated the early response to chemotherapy rather than the survival of the patients. Early response is important, because patients with aggressive lymphoma who respond poorly to intensive chemotherapy seem to have a short survival period. This is supported by a report that early application of autologous bone marrow transplantation does not improve outcome in patients with aggressive NHL who respond slowly to first-line CHOP chemotherapy [24].

In regard to the relation between the response to the anticancer drugs and p53 gene mutations or p53 overexpression, human lymphoma cell lines with decreased sensitivity to the drugs [25] and carcinoma with resistance to cisplatin-based chemotherapy [26] were reported. In this study, the expression of p53 at admission showed no significant relation with early response to chemotherapy in aggressive NHL. In low-grade NHL, all patients were p53-negative; however, transformation from a follicular to diffuse pattern may induce p53 overexpression.

In spite of overexpression of p53, only one patient among three examined by PCR-SSCP showed a conformational change and alteration in exon 7. This patient was refractory to chemotherapy. However, of the other two patients without p53 gene alterations, one relapsed after CR and another was in PD. Mutation of p53 gene might occur in exons other than exon 5 through 8.

We hypothesized that tumor cells with p53 mutations entered the cell cycle, resulting in an increase in the number of S-fraction and PCNA positive cells, because these cells resisted growth arrest and apoptosis. However, we could not find any correlation between the expression of p53 and PCNA. Although PCNA staining analyses are more popular in carcinoma than lymphoma, PCNA and Ki-67 scores and S-phase fraction correlated closely with the proliferative activity of intermediate-grade NHL [27]. In the present study, the expression of PCNA also showed no significant relation to the International Index or early response to chemotherapy in aggressive NHL. High PCNA-positivity does not seem to be a marker of poor prognosis in aggressive NHL. Similarly, in low-grade NHL, the expression of PCNA was not evaluated.

In conclusion, the overexpression of p53 and PCNA was not a marker of a poor response to chemotherapy or of high risk under the International Index. And the ex-

pression of p53 by immunostaining might depend on factors other than p53 gene mutations. Clinical analysis using immunohistochemical staining may not play an important role in prognosis in non-Hodgkin's lymphoma.

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